Maternal Fermented Diet Drives Early-Life Gut Health by Boosting Milk L-Glutamine and Gut *Lactobacillus Reuteri*

Cheng Wang
Zhejiang University College of Animal Sciences
Siyu Wei
Zhejiang University College of Animal Sciences
Mingliang Jin
Zhejiang University College of Animal Sciences
Yizhen Wang (11617015@zju.edu.cn)
Zhejiang University College of Animal Sciences https://orcid.org/0000-0002-4210-5412

Research

Keywords: maternal nutrition, MFD, fat metabolic pathway, Faecal microbiota transplantation, microbiota

Posted Date: October 20th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-951417/v1

Abstract

Background

Knowledge is growing on how maternal nutrition shapes neonatal health. However, the effects of maternal factors on gut health in offspring and how the maternal fermented diet (MFD) affects it remain elusive. Here, we applied *in vivo* and *in vitro* models to investigate the impacts and the underlying mechanism of the MFD on early-life gut health.

Results

The MFD remarkably improved the performance of both sows and piglets, significantly increased the abundance of maternal gut *Lactobacillus*, *Succiniclasticum* and enhanced protein and fat metabolic pathways. α-guanidinoglutaric acid, hippuric acid, L-glutamine (GLN), and pyridoxic acid, as well as glutamine metabolism in the milk were notably improved by the MFD. In addition, the MDF significantly accelerated the maturation of the gut microbiome in early life, increased the abundance of gut *Lactobacillus*, and the microbial functions of amino acid-related enzymes and glucose metabolism on the weaning day. Notably, the MFD reduced the susceptibility to lipopolysaccharide (LPS)-induced colonic inflammation in offspring. Faecal microbiota transplantation (FMT) suggested that the MFD might play critical roles in early-life gut health by mediating maternal gut microbiota. Multi-omics analysis and *in vivo* and *in vitro* assays revealed maternal *Lactobacillus reuteri* (LR) and milk GLN were two main maternal effectors that contributed to the gut health of offspring. The properties of LR and GLN in alleviating LPS-induced colonic inflammation by inhibiting the phosphorylation of p38 MAPK and JNK were further revealed in mice and cells.

Conclusions

These findings provide the first data revealing that the MFD drives early life gut microbiota development and ameliorated the colonic inflammation by boosting milk L-glutamine and gut *Lactobacillus reuteri*. These fundamental data might provide references for modulating maternal nutrition to prevent early life gut inflammatory bowel disease and enhance gut health.

Introduction

Early life is a critical period during which the gut microbiome, physical barrier, and immunity are established in infants. There are increasing evidences that the maturation and health of the newborn gut may exert important properties in programming health and disease later in life [1]. Disturbing early-life gut development has been found to be associated with the occurrence of gut diseases, including inflammatory bowel disease (IBD) in both children and adults [2, 3, 4].

Maternal milk and gut microbiota are two main factors which contribute to early postnatal gut development [5, 6]. Maternal milk affects the maturation of the gut in infants by supplying not only basic

nutrients, but also bioactive components such as immunoglobulin, oligosaccharides, short chain fatty acids, and antimicrobial peptides, as well as microbes [7]. Maternal gut microbiota shapes the early-life gut microbiome through the entero-mammary axis or creating environment microbiome in the home [8]. In addition, maternal gut microbiota can drive early postnatal innate immune development and metabolic phenotype [9, 10].

Diets can dramatically and rapidly affect the gut microbiome [11]. The fermented diet, as one type of functional diet, not only contains foundational nutrients (carbohydrates, proteins, and fats), but also consists of probiotics, prebiotics, and microbial metabolites [12]. Many studies have reported the benefits of the fermented diet on gut health, while the effects of the maternal fermented diet (MFD) on gut health in offspring remains unstudied [13].

In the present study, *in vivo* and *in vitro* models were used to investigate how the MFD drives early-life gut health by applying multi-omics, faecal microbiota transplantation (FMT), and lipopolysaccharide (LPS)-induced colonic inflammation. These results will expand our knowledge of how maternal nutrition influences the gut health of infants during early life and provide underlying strategies to intervene in gut development during early life with lasting consequences.

Materials And Methods

Production of maternal fermented diet

The fermenting substrate consisted of corn, soybean meal, and wine lees (2:2:1). Sterile water was added to achieve an optimal 40% moisture content. *Bacillus subtilis* CW4 (NCBI Accession No. MH885533, 1×10^8 CFU/g) and *Enterococcus faecalis* CWEF (NCBI Accession No. MN038173, 1×10^8 CFU/ g) were included in the diet to promote fermentation over the course of 3 days. The nutritional values of MFD are shown in Table S1.

Experimental design

Sixty Yorkshire × Landrace sows from the week before parturition to the day of weaning were randomly divided into three groups (Fig. 1a): (i) CON group (control diet, n = 20), (ii) MFD group (basal diet + 10% MFD, n = 20) and (iii) PROB group (basal diet + equal amount of *B. subtilis* and *E. faecalis*, n = 20). For the PROB group, 3.4 g kg^{-1} of *B. subtilis* powder and 1.3 g kg^{-1} of *E. faecalis* powder were supplied in water. The diets had same amounts of crude protein and digestive energy. The ingredients and nutritional values are shown in Table S2. The sow feces were collected on day 28 and the piglet feces were obtained on day 7, 14 and 28.

To examine the effects of MFD on the resistance of offspring to colonic inflammation, piglets were challenged with LPS (Sigma-Aldrich, St. Louis, MO, USA) (Fig. 1b). Healthy piglets with similar weights were randomly selected from CON, MFD, and PROB groups. Six piglets in each group were intraperitoneally injected with 10 mg/kg of LPS. The other six piglets in the CON group were

intraperitoneally injected with an equivalent amount PBS. Piglets were divided into CON, LPS, MFD + LPS, and PROB + LPS groups, slaughtered on day 21, and sampled after 12 h.

To further investigate whether MFD could affect early-life gut health by regulating the maternal gut microbiome, we performed an FMT assay using mice (Fig. 1c). Twenty-four healthy pregnant female C57BL/6J mice were allotted into four groups with six mice per group. After 2 weeks of broad-spectrum antibiotic treatment, the dams were intragastrically administered a sow fecal microbiota suspension every other day. At the time of weaning on day 21, the dams and pups were intraperitoneally injected with 10 mg/kg LPS, and the feces and colon samples were collected 12 h later. Dams and pups were divided into CON-trans, CON-trans + LPS, MFD-trans + LPS and PROB-trans + LPS groups.

Mice were further used to verify the properties of maternal effectors *in vivo* (Fig. 1d). Thirty healthy 4-week-old C57BL/6J male mice were allotted into five groups with six mice per group. They were pre-fed for one week and treated with 1×10^8 CFU/d LR (*Lactobacillus reuteri*, ATCC 53608), 300 mg/kg GLN (Solarbio, Beijing, China), or a combination of both agents (MIX) for 21 days. Thereafter, the mice were intraperitoneally injected with 10 mg/kg LPS for 12 h and the feces and colon samples were collected. The groups were CON, LPS, LR + LPS, GLN + LPS, and MIX + LPS.

Porcine macrophage 3D4/2 and human Caco-2 cells were selected to investigate the effects of maternal effectors on LPS-induced inflammation *in vitro* (Fig. 1e). Cells were treated with 10 μg/mL of LPS or an equivalent amount of PBS for 12 h after treatment (1×10^8CFU/mL LR or 2.5 mM GLN) for 8 h. Then the total protein and RNA of the cells were collected.

16S sequencing and data analyses

Microbial DNA was obtained from fecal homogenates using the E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA). To ensure that no contamination had occurred, the concentration and purity of the DNA samples were measured with the NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, MA, USA) and examined by electrophoresis using 1% agarose gels. The primer pairs 515F (5'-GTGCCAGCMGCCGCGG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were selected to amplify the 16S rRNA gene using the Illumina MiSeq platform at Shanghai Majorbio Biopharm Technology Co., Ltd. (Shanghai, China).

The reads were preprocessed, denoised, quality controlled, and merged in dada2 [14]. UPARSE 7.1 and QIIME2's q2-feature-classifier plugin were used to cluster the operational taxonomic units (OTUs) with 97% and 100% similarity cutoffs [15], respectively, and chimeric sequences were identified and removed. The taxonomies from phylum to genus levels were assigned by the Greengenes database [16], and the OTUs were analyzed against the Silva database. Sequences of interest were further confirmed through NCBI platforms, and highly similar sequences were selected based on the optimization of the BLAST algorithm. Diversity analyses were performed using Qiime1 and Qiime2 [17]. In addition, predicted microbial metabolic functional annotations were obtained using Kyoto Encyclopedia of Genes and Genomes (KEGG) under PICRUSt2 (https://github.com/picrust/picrust2) [18].

To identify age-related bacteria in the gut microbiome, a random forest model was used to train the data in the control group, and then the age of the microbial community was modeled for those same taxa in all groups. The maturation index of the intestinal microbiota was calculated.

Metabolomic profiling by LC-TOF/MS

Milk samples randomly selected from six sows in each group were used for extraction and sent for metabolomic analysis. The samples were extracted to obtain the supernatants. An equivalent volume was aliquoted from each sample, mixed to prepare the QC sample and dried in a vacuum concentrator. In addition, methoxymethyl amine salt was mixed with the dried sample, and bis(trimethylsilyl)trifluoroacetamide was added. After cooling to room temperature, fatty acid methyl ester was added to each sample and mixed. Ultra-performance liquid chromatography (1290 Infinity series UHPLC System, Agilent Technologies, Santa Clara, CA, USA) was applied for LC-TOF/MS analysis. A UPLC BEH amide column (internal diameter, 2.1 × 100 mm, 1.7 μm, Waters, Milford, MA, USA) was used for separation. The obtained data were applied for principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). R2 and Q2 were used to evaluate the quality of PCA and OPLS-DA models. Differential metabolites were identified with variable importance projection (VIP) > 1.0 and $P < 0.05$. To further interpret the biological significance of the differential metabolites, metabolic pathway analyses were performed using an online analysis platform in MetaboAnalyst 4.0 (https://www.metaboanalyst.ca/). The different milk metabolites were present at Table S3

Intestinal morphology and histology

Approximately 2 cm of the proximal colon was fixed in 4% paraformaldehyde. Colonic tissues were stained with hematoxylin and eosin using the Leica DM3000 Microsystem, and Leica Application Suite 3.7.0 (Leica, Wetzlar, Germany) was applied to observe the colonic morphology. Histological scoring was performed as described by Shahanshah et al. [19].

A suitable size of colonic tissue was placed in 2.5% glutaraldehyde and fixed overnight. The fixed sample was washed with PBS, soaked in 1% osmic acid solution for 2 h and washed with PBS. Subsequently, the sample was dehydrated in different concentrations of alcohol and dehydrated twice with absolute ethanol. The sample was transferred to a 1:1 volume mixture of ethanol and isoamyl acetate for 30 min and then transferred to pure isoamyl acetate for 1 h. After the critical point was dried, the gold-platinum film was coated and observed with a field emission scanning electron microscope.

The colonic tissue fixed by the osmic acid solution was washed with PBS and dehydrated in different concentrations of alcohol and acetone. The sample was placed in a mixture of pure acetone and embedding agent for 1 h, transferred to a mixture of pure acetone and embedding agent for 3 h, and finally transferred to embedding agent for 12 h. The embedded sample was heated and then sectioned with an ultra-thin microtome. Finally, the sections were stained and treated with uranyl acetate and alkaline lead citrate. A suitable field of view was identified and imaged with a JEM-1011 transmission electron microscope (JEOL USA, Peabody, MA, USA).

Immunofluorescent staining and TUNEL

Colonic slides were incubated with antigen-recovering solution (Vector Laboratories, Inc. Burlingame, CA, USA) for 15 min, and the non-specific binding was blocked with 5% BSA. Slides were transferred to anti-ZO-1 and anti-β-catenin primary antibodies (ab96587 and ab32572, Abcam, Cambridge, MA, USA) at 4°C overnight. After washing with PBS, slides were incubated with fluorescent dye-conjugated secondary antibodies (Abcam) for 1 h and protected from light. Finally, 4, 6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus. Sections were examined with a DM5000 B fluorescent microscope (Leica, Wetzlar, Germany).

After colonic sections were deparaffinized, DNase-free proteinase K (20 μg/mL) was applied and sections were incubated at 37°C for 20 min. After washing with PBS, the TUNEL detection solution was added dropwise, and sections were incubated in a 37°C incubator for 2 h. After adding the stop solution to complete the reaction, sections were washed with PBS. Thereafter, the streptavidin-HRP working solution was added dropwise, sections were incubated for 30 min. Sections were washed with PBS, followed by the addition of 0.2–0.5 mL of DAB and incubation at room temperature. The specific time was determined according to the degree of color development and images were acquired for analysis.

Western blotting

Total proteins were obtained using the Protein Extraction Kit (KeyGen BioTECH, Nanjing, China). SDS-PAGE separates proteins of different sizes and electroporates them onto PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% skimmed milk and incubated with anti-ZO-1, anti-Occludin, anti-Claudin-1, anti-Cleaved capase3, anti-Bax, anti-Bcl-2, anti-iNOS, anti-Arg1, anti-p-p38, anti-p38, anti-p-JNK, anti-JNK, anti-p-ERK1/2, anti-ERK1/2, and anti-β-actin antibodies at 4°C overnight. After washing with TBST, membranes were transferred to secondary antibodies for 1 h. Protein bands were visualized with an ECL Assay Kit (Biosharp, Hangzhou, China) and measured with Image J software (NIH, Bethesda, MD, USA).

ELISA assay

Approximately 0.1 g of colonic tissue was homogenized after milling at 12000 × g for 10 min at 4°C. Cytokine concentrations in supernatants were measured with ELISA Kits (Jiangsu Meibiao Biological Technology Co., Ltd., Jiangsu, China).

In vitro fermentation

Approximately 1 g of pre-digested MFD was suspended in 25 mL of sterile fermentation medium and boiled for 10 min. The boiled MFD solution was introduced into the anaerobic chamber, cooled down to room temperature, and reduced for 2 h. Thereafter, 5 mL of sterile fermentation medium was combined with 0.1 g of faecal sample and homogenized, followed by the mixing of 2.5 mL of the homogenized faecal suspension and 2.5 mL of the MFD solution (final MFD concentration, 1%; final fiber concentration, 2%) and incubation under anaerobic conditions at 37°C with 125 rpm shaking for 14 h. All fermentation

steps were conducted in an anaerobic chamber (Bactron, Sheldon Manufacturing, Inc., Cornelius, OR, USA) in an anaerobic environment containing 5% CO_2, 5% H_2, and 90% N_2.

Quantitative real time PCR and absolute quantification of *L. reuteri*

Primers were used to measure the copy numbers of the 16S rRNA gene of specific bacteria (V3-V4). The *Lreu* gene was selected to analyze the abundance of the *L. reuter* strain. Forward and reverse primers were used as follows: 5'-CAGACAATCTTTGATTGTTTAG-3' and 5'-GCTTGTTGGTTTGGGCTCTTC-3' [20]. To obtain a standard curve, plasmids containing target DNA PCR fragments of 16S rRNA and *Lreu* genes were diluted 10-fold. qPCR reactions and analyses were conducted as previously described [20]. The $^{\Delta}$Ct method was used to calculate the copy numbers of *Lreu* genes from the standard curve of 16S rRNA.

Cell culture

Caco-2 and 3D4/2 cells were obtained from ATCC Cell Bank (Shanghai, China). DMEM-F12 medium supplemented with 10% fetal bovine serum and double antibiotics at 37°C was used. Cells were incubated in an incubator containing 5% CO_2.

In vitro gut paracellular permeability

Approximately 100 μL of 4-kDa FD4 (1 mg/mL) was added to the apical chamber after the last TEER measurement. The plates were placed at humidified incubator for 30 min. The prepared FD4 solution was serially diluted (5, 10, 20, 40, 80, 160, 320, 640, 1,280 ng/mL). Subsequently, the level of FD4 in the basolateral chamber was measured (excitation wavelength, 492nm; emission wavelength, 520nm).

Transepithelial electrical resistance (TEER)

Transwell filters (Corning, NY, USA) were used to culture Caco-2 cells. When the TEER stabilized, cells were used for subsequent experiments. The TEER was determined using the Millicell ERS voltohmmeter (Millipore, Burlington, MA, USA) at 0, 2, 4, 6, 8, 10, and 12 h after LPS stimulation. Changes were analyzed as a fold of TEER at 0 h.

Statistical analysis

All data were presented as means ± SEM as analyzed by SPSS 26.0 (SPSS Inc. Chicago, IL, USA). The “corrplot” package in R (R Core Team, 2014) was used to obtain the Pearson correlation coefficient and significance. Differential gut microbes were verified by the ANOVA test and Benjamini–Hochberg FDR adjusting of STAMP (version 2.1.3). The contribution of maternal factors on gut microbiota in offspring was calculated by variance partitioning analysis (VPA) using the “varpart” package of R.

Results

MFD improved the performance of sows and piglets

Swine (*Sus scrafa*) is a superior animal research model used to study the impact of the gut microbiome on host gut health because the physiological functions, gut structure, and microbiota of pigs are similar with human beings, and their diets are easily manipulated. The impact of the MFD on the performance of sows and piglets was preferentially examined (Table S4). As shown in Table S4, the MFD significantly improved the average daily feed intake and milk yield in sows. Piglets in the MFD group showed a significant increase in weaning weight gain and a decrease in diarrhea incidence. PROB tended to promote the average daily feed intake and milk yield in sows, as well as piglet weaning weight gain and it notably decreased diarrhea incidence.

MFD changed the composition of the sow gut microbiome and milk metabolome

On the maternal aspects, the effects of the MFD on the gut microbiome and milk metabolome in sows were investigated. Compared with the CON group, the α diversity of the maternal gut microbiome in the MFD group tended to increase, whereas it significantly increased in the PROB group (Fig. 2a). Principal component analysis showed that the β diversity of the maternal gut microbiome was significantly different among the groups (Fig. 2b, $P < 0.001$). The heatmap of the most differentially abundant genera revealed that the abundance of gut *Lactobacillus* and *Succiniclasticum* significantly increased in the MFD group, whereas the abundance of *Mitsuokella* and *Erysipelotrichaceae* increased in the PROB group(Fig. 2c, LDA > 3.0). The MFD enhanced the functions of maternal gut microbiota related to the metabolism of carbohydrates, proteins, and fats (Fig. S1a). Furthermore, 2007 effective peaks were obtained in the metabolome results of milk among the three groups. The sPLS-DA plot of cluster analysis showed that the clusters between the three groups were clearly separated (Fig. 2d). Among 43 differential metabolites, the MFD significantly increased the concentration of α-guanidine glutaric acid, hippuric acid, L-glutamine (GLN), and pyridoxic acid in the milk, while significantly reduced the aroma leaf geraniol, 1-stearoyl-sn-glycerol-3-choline phosphate, deoxycytidine, butyl betaine hydrochloride, and 5-methylcytosine (Fig. 2e, VIP > 1.0, $P < 0.05$). The metabolites of the top 15 VIP values with significant differences showed the differential metabolic pathways of milk (Fig. 2f). Phenylpropane metabolism; phenylalanine, tyrosine and tryptophan biosynthesis; aminoacyl biosynthesis; D-glutamine and D-glutamate metabolism; and alanine, aspartic acid and glutamate metabolism were significantly different among the groups. Among them, D-glutamine and D-glutamate metabolism, aminoacyl biosynthesis, and phenylpropane metabolism were the top three significantly enriched metabolic pathways. The significantly different metabolites and metabolite-related metabolic pathways are shown in Fig. S1b and c.

MFD improved the longitudinal and horizontal assembly of early life gut microbiota

The gut microbiome is essential for gut development and health. Therefore, the effects of the MFD on the longitudinal and horizontal assembly of the gut microbiome in offspring were investigated. The gut microbiota of piglets changed over time, and the α diversity continued to increase over time (Fig. 3a). Consequently, the functions of the gut microbiome of offspring changed (Fig. S3a). MFD significantly increased the α diversity and changed the β diversity of the early-life gut microbiome in late lactation (Fig.

3a, b). Notably, the MFD significantly reduced the microbial functions of bacterial toxins and LPS biosynthesis in late lactation (Fig. S3b). The MFD firstly increased and then maintained the abundance of gut *Firmicutes* in piglets, whereas other groups showed an increasing and then a decreasing trend (Fig. 3c). Interestingly, the maturation patterns of the gut microbiome, as indicated by the microbiota maturation index of each group, were different (Fig. 3d). The gut microbial development curve of the MFD was the fastest group reaching a plateau, as early as day 14 of lactation. To better understand how the MFD significantly affects interactions between microbes in the early-life gut microbiome, the co-occurrence network of gut microbial interactions at different time points was analyzed. The abundance and edge number of *Lactobacillus*, *Bifidobacterium*, *Prevotella*, *Dallella*, and *Oscillatoria* were higher than those of other genera (Fig. S2). The gut microbiota and functions of the three groups of piglets on day 28 were further analyzed. Compared to the other groups, the MFD increased the abundance of gut *Lactobacillus* and *Prevotella* in offspring (Fig. 3e, LDA > 2.5), and metabolic processes such as those involving amino acid-related enzymes and glucose (Fig. S3c).

MFD reduced the susceptibility of offspring to LPS-induced colonic inflammation

Neonates are sensitive to pathogenic infection due to the fact that their digestive system is not yet completely developed, thereby causing colonic inflammation and IBD. To assess the resistance of offspring to colonic inflammation, piglets were challenged with LPS. The results of hematoxylin and eosin staining and electron microscopy revealed that the MFD significantly alleviated the LPS-induced injury of the piglet colon, that is, the morphology of the microstructure (Fig. 4a). Similarly, the MFD restored the decreased protein levels of ZO-1, β-catenin, Occludin, and Claudin1 induced by LPS (Fig. 4b, c). In addition, the increase in the colonic apoptotic index and Bcl2 expression, and the decrease in cleaved caspase 3 protein expression was mitigated in the colon of the MFD group (Fig. 4d, e). In terms of colonic inflammation, the results showed that the MFD reduced the decrease of Arg1 and the increase of iNOS protein expression in the piglet colon caused by LPS (Fig. 4f). The MFD alleviated the LPS-induced increase in IL-1β and IFN-γ levels and the decrease in IL-10 and TGF-β levels in the colon (Fig. 4g). The overall protective effects in the MFD group were better than those in the PROB group.

MFD programed early-life gut health by altering the maternal gut microbiome

To verify whether the MFD improves early-life gut health by regulating the maternal gut microbiome, the fecal microbiota transplantation (FMT) assay was performed. Fecal microbiota from sows of CON, MFD, and PROB groups were transplanted to antibiotic-treated dams for 4 weeks. The dams and pups were intraperitoneally injected with LPS on the day of weaning. The β diversity of the gut microbiome of dams transplanted with sow fecal microbiota was significantly different after LPS treatment (Fig. 5a, P = 0.049). The abundance of gut *Lactobacillus* and *Clostridium* of dams in the MFD-trans group was remarkably enriched compared to that of the other groups, whereas the abundance of *Streptococcus*, *Pantoea*, and *Helicobacter* was enriched in CON, LPS, and PROB groups, respectively (Fig. 5b, LDA > 3.0). With regard to the morphology of the gut, maternal FMT from the MFD group alleviated LPS-induced colonic damage and decreased ZO-1, Occludin, and Claudin1 protein levels (Fig. 5c, d). The results of

pups after LPS treatment were similar to those of dams. Although there was no difference in the weaning weight and weaning weight gain of pups among the groups (Fig. S4a). Maternal FMT remarkably differentiated the β diversity of gut microbiota in pups after LPS challenge (Fig. 5e, $P = 0.007$). The abundance of gut *Firmicutes*, *Lactobacillus*, *Bacteroides*, and *Streptococcus* in pups of the MFD-trans group was notably enriched than that in the other groups after LPS treatment, whereas the abundance of *Anaerobacillus*, *Sutterella*, and *Arthrobacter* was enriched in CON-trans, CON-trans+LPS, and PROB-trans+LPS groups, respectively (Fig. 5f, LAD > 2.5). The LPS-induced damage to the morphology of the colon, the improved gut permeability, and the decline in Occludin and Claudin1 protein levels in pups were restored in the MFD transplanted group (Fig. 5g, h; Fig. S4b). The transplantation of fecal microbiota from the MFD mitigated the LPS-induced increase in IL-6 and TNF-α and the decrease in IL-10 in the colon of pups (Fig. S4c). The overall colonic status of both dams and pups in the MFD group after LPS challenge was better than that of the PROB transplanted group.

Maternal gut *Lactobacillus reuteri* and milk L-glutamine were two effectors that enhanced gut health in offspring

Maternal gut microbiota and milk play critical roles in early-life gut health. Neonatal gut microbiota are essential in the maintenance of gut health. Therefore, correlation analysis was performed to investigate the effectors of maternal gut microbiota and milk metabolites that affected the gut health of offspring. The results indicated that there was a significant positive correlation between gut *Lactobacillus* in sows and piglets (Fig. 6a). A significant positive correlation between GLN in the milk metabolite fraction and gut *Lactobacillus* in piglets was also identified (Fig. 6b). The interpretation rates of sows' gut microbiota and milk metabolites to piglets' gut microbiota revealed by VPA interpretation analysis were 0.86% and 1.36%, respectively (Fig. S5a). The common interpretation rate of both sows' gut microbiota and milk metabolites reached 93.52%, and the unexplainable factor accounted for 4.26%. Additionally, correlation heat map analysis of piglet gut microbiota and the piglet apparent index showed that gut *Lactobacillus* was significantly positively correlated with piglet weaning weight gain, serum growth hormone, and TGF-β concentration, and negatively related to the diarrhea rate, zonulin expression, and TNF-α concentration (Fig. 6c). Besides, the relative abundance of gut *Lactobacillus* of sows and piglets and the concentration of GLN in the milk metabolite fraction were enriched in the MFD group (Fig. 6d). The absolute concentration of milk GLN increased in the MFD group (Fig. 6e). The most differential sequences of gut *Lactobacillus* in sows and piglets were blasted with the NCBI database, and it was found that they were all *Lactobacillus reuteri* (LR) (Fig. S5b). Furthermore, the absolute quantification of LR in sow and piglet feces was enriched in the MFD group (Fig. 6f). The *in vitro* fermentation of maternal fecal microbiota from the CON group indicated that the MFD could enhance the proliferation of LR compared to the CON diet (Fig. 6g).

LR and GLN alleviated LPS-induced colonic inflammation by suppressing phosphorylation in the p38 MAPK/JNK pathway

The effects of LR and GLN on LPS-induced colonic inflammation were further examined in mice and cells. No difference was found in mouse weight gain among the groups (Fig. S6a). The results of β diversity showed that the structures of gut microbiota in mice were clearly distinguishable among the groups (Fig. 7a, $P < 0.001$). LR and GLN (MIX) alleviated the LPS-induced decrease in the abundance of *Lactobacillus reuteri* (Fig. 7b, LDA > 3.0). MIX restored the morphology of the damaged colon and increased the gut permeability caused by LPS (Fig. 7c, Fig. S6b). MIX treatment alleviated the LPS-induced decrease in ZO-1, Occludin, and Claudin 1 protein levels (Fig. 7d). MIX restored the LPS-induced increase in IL-6 and TNF-α concentrations and the decrease in IL-10 and TGF-β concentrations (Fig. 7e). The effects of MIX were better than either treatment alone.

The optimal treatment concentration of LR and GLN was investigated using Caco-2 and 3D4/2 cells in cell viability and cytotoxicity assays (Fig. S7c, d). LR and GLN treatment diminished the LPS-induced decrease in the survival rate of 3D4/2 cells (Fig. S7e). Compared with the CON group, LPS treatment reduced the expression of Arg1 in 3D4/2 cells and increased the expression of iNOS. LR and GLN ameliorated this phenomenon (Fig. S7f).

LR and GLN treatments alleviated the LPS-induced significant decrease in the TEER values of Caco-2 cells, and the TEER values of MIX and LPS treatment had the largest difference at 12 h (Fig. 7a). LR and GLN significantly resisted the increase in FD4 content caused by LPS (Fig. 7b). Furthermore, LR and GLN alleviated the LPS-induced decrease in the viability of Caco-2 cells (Fig. S7g). LR and GLN restored the LPS-induced decrease in ZO-1, Occludin, Claudin 1 and Bcl-2 expression, and improved the expression of cleaved caspase 3 and Bax (Fig. 7c). Meanwhile, LR and GLN mitigated the LPS-induced increase in the phosphorylation of p38 MAPK and JNK proteins (Fig. 7d). Additionally, the activators of p38 (S2266), JNK (S7409), and ERK1/2 (S1013) were used to explore the effects of MIX on the LPS-induced expression of MAPK-related pathway proteins. The results showed that the three activators effectively activated the respective proteins of the MAPK pathway (Fig. S7i). Remarkably, p38 MAPK and JNK activators significantly reduced the suppressive effects of MIX on alleviating the LPS-induced decrease in the cell survival rate and tight junction protein expression and the increase in pro-apoptotic protein expression, while ERK1/2 activator had no effect (Fig. 7e). The effects of MIX on alleviating LPS-induced inflammation was better than those of LR and GLN alone.

Discussion

The colonization of early-life gut microbiota is essential for gut development and contributes to short- and long-term health outcomes [21]. Maternal nutritional states play fundamental roles in offspring gut health programming [22]. Fermented diets are functional foods that can positively regulate gut health [23]. However, there is little evidence to explain how the MFD manipulates gut development and health in offspring. Here, MFD-induced milk GLN and gut LR were found to contribute to early-life gut health by improving the establishment of early gut microbiome, attenuating LPS-induced gut inflammation by suppressing the phosphorylation of p38 MAPK/JNK.

Gut development in offspring is largely affected by the maternal gut microbiome and breast milk [24]. Therefore, the MFD-induced maternal gut microbiome and milk metabolome were analysed. There is little information on the effects of diet on the maternal gut microbiome, although it is important during pregnancy and lactation [25]. Roytio et al. reported that obese mothers who consumed sufficient amounts of fiber during pregnancy showed low-grade inflammation, a reduced abundance of gut *Bacteroidetes*, and a higher degree of gut microbial richness [26]. Mandal et al. found that low maternal intake of fat-soluble vitamins was related to increased gut microbial diversity and reduced abundance of *Proteobacteria* [27]. Only one study indicated that the gut microbial community in lactating women was related to macronutrient and micronutrient intake [28]. In the present study, the MFD increased the α diversity of the maternal gut microbiome and reduced the abundance of *Enterobacteriaceae* and *Klebsiella*. The low pH and probiotics in the MFD may have prevented pathogenic infection and enhanced gut health. The MFD significantly promoted the abundance of gut *Lactobacillus* and *Succinobacterium*. *Lactobacillus* has been widely studied because of its ability to enhance gut barrier function, balance the gut microbiome, modulate the innate immune system, and prevent the colonization of pathogenic bacteria, thereby benefiting host health [29]. *Succiniclasticum* can utilize succinate and produce propionate to exert benefits to gut health [30]. The results suggest that MFD not only inhibits pathogens, but also plays an important role in the modulation of gut commensal microbiota. Furthermore, most microbes with functions in the metabolism of carbohydrates, amino acids, and fats were enriched in the MFD group, indicating that the MFD might improve gut health in offspring by providing more nutrients to sows. The results of the analysis of the milk metabolome indicated that several amino acids and organic acids were significantly up-regulated by the MFD. Additionally, the metabolic function of glutamine in the milk metabolome was enhanced. Milk metabolites can promote microbial-dependent growth in infants [31]. Wu et al. reported that breast milk from pathological mothers could impair the growth of newborns [32]. MFD-induced milk metabolites and related metabolic processes provide great possibilities to improve gut health in offspring. Thus, the impact of the MFD on gut health and growth performance in offspring was attributed to the manipulation of maternal nutrition, as a consequence of the comprehensive effects of the vertical transmission of microbes and milk metabolites.

Studies have indicated that maternal diets during pregnancy can affect the gut health and risk of pathogen infection in offspring in early and late life [33–36]. In this study, the effects of the MFD on the longitudinal and horizontal assembly of the gut microbiome in offspring during lactation were investigated. The MFD significantly improved the diversity and abundance of gut *Firmicutes* in offspring at a late stage of lactation. The results revealed that the effects of the MFD on the development of the gut microbiome depended in a time manner. The MFD significantly reduced the significance of LPS biosynthesis and bacterial toxins with time. Interestingly, the MFD accelerated the maturation of the gut microbiome in offspring. Additionally, the microbiota on day 14 had the highest number of correlations and level of abundance, indicating that they may have undergone remarkable changes and play an important role in the development of the gut microbiome in offspring [37]. Furthermore, the number of gut microbial interaction edges decreased as the offspring aged, demonstrating gut microbiota shifted from the initial significant changes to a steady state during lactation. The structural and functional dynamics

of the gut microbiome in offspring can be used as an indicator of growth and health [38]. A mature gut microbiome in suckling offspring is beneficial to host growth and development and prepares animals for weaning [39]. Therefore, the MFD may improve gut development and health in offspring by promoting the maturation of the gut microbiome. Horizontally, the MFD increased the abundance of gut *Lactobacillus* and *Prevotella* as well as amino acid-related enzyme function and glucose metabolism in piglets on day 28. One human study investigated the associations between maternal diet and gut microbiota in breastfeeding infants [40]. Babakobi et al. found no correlations between maternal diet and the gut microbiome in offspring due to the difficult specific assessment of maternal dietary consumption during lactation. Two studies reported the correlations between maternal fiber consumption and the gut microbiome during pregnancy and lactation in pigs. A decreased abundance of gut *Enterococcus* and an increased abundance of *Clostridiaceae* were found in piglets from sows fed a fiber-rich diet [41, 42]. The different results may be attributed to the differences in intake volume, with proper supplementation volumes having the potential to enhance the effects of the MFD. In addition, different fermenting strains used to produce the MFD could have affected the results [43]. To take advantage of their combined probiotic properties, *Bacillus* and *Lactobacillus* were used as the inoculum in the present study, which promoted the development of the gut microbiome in offspring by maternal intervention.

Piglets were challenged with LPS to examine the effects of the MFD on the resistance of offspring to colonic inflammation. The MFD significantly diminished LPS-induced morphological injury to colonic epithelial cells. The gut epithelial barrier consists of epithelial cells and intercellular junction proteins that play important roles in preventing inflammation and infection [44]. An increase in the abundance of beneficial microbes in the gut can improve the integrity of the intestinal barrier [45]. Cheng et al. [46] transplanted healthy Jinhua pig faecal microbiota into K88-infected piglets that were found to have the improved intestinal morphology, the reduced intestinal permeability and the enhanced expression of mucin and mucosal tight junction proteins. The MFD improved the establishment of the gut microbiome in piglets and increased the abundance of *Lactobacillus*. These results demonstrated that the MFD benefited the gut microbiome in offspring to mitigate the LPS-induced impairment of colonic physical barriers. Cytokines are involved in the regulation of gut homeostasis and immune function [47]. In the present study, the MFD restored the cytokine disorder in offspring. Our previous study reported that the serum levels of anti-inflammatory cytokines in sows was enhanced by the MFD [48]. Maternal immune factors can be transmitted vertically to offspring [49, 50]. Liu et al. [51] demonstrated that the concentration of IL-10 in maternal serum and faeces increased, those of IL-6 and TNF-α decreased, and that of IL-6 in serum of offspring also decreased by adding different dietary supplements to feed sows. A study has also shown that the improvement of colitis was mainly due to the decrease in inflammatory cytokine levels and the increase in anti-inflammatory cytokine levels [52]. Thus, the MFD may ameliorate LPS-induced colonic cytokine disturbances in offspring by improving the immune status of sows. Macrophages consist of pro-inflammatory macrophages (M1 type) and anti-inflammatory macrophages (M2 type), which play vital roles in the inflammatory response [53]. The MFD significantly alleviated LPS-induced pro-inflammatory colonic macrophages in offspring, which may be one of the anti-inflammatory mechanisms. Apoptosis is a type of programmed cell death that involves the activation of caspases,

which is often accompanied by impairment of the gut barrier [54]. Therefore, the MFD can diminish apoptosis in the colon of LPS-challenged piglets and promoted epithelial barrier function.

FMT is a critical technique in the investigation of the functions of gut microbiota [55]. To further investigate whether the MFD affected early-life gut health by regulating the maternal gut microbiome, sow faecal microbiota were transplanted to broad-spectrum antibiotic-treated dams. FMT from the MFD notably alleviated the LPS-induced decrease in α diversity and gut *Lactobacillus* in both dams and pups. Accordingly, FMT from the MFD significantly mitigated the LPS-induced colonic inflammation in dams and pups. Overall, these results are consistent with those of pigs, verifying that the MFD might improve the gut health of offspring by positively modulating the maternal gut microbiome.

Notably, the effects of the MFD were better than those of the PROB in both maternal and offspring results. This might have been attributed to a high level of probiotics in the MFD, which could positively shape the gut microbiome. At the same time, the MFD contains bioactive compounds, like hydrolysed small peptides, which could enhance physical barrier function, activate host immune cells, and improve gut functions. In addition, microbial metabolites in the MFD, such as organic acids, enzymes, and antimicrobial peptides, also exist and exert their properties [12].

To identify the main maternal components that affect gut health in offspring, correlation analysis and absolute quantification were conducted. The results of correlation analysis indicated that the maternal gut LR promoted the abundance of the offspring gut LR, thereby improving gut health and the growth index. In addition, the relative and absolute abundance of LR *in vivo* and *in vitro* and milk GLN were significantly enriched in the MFD group, indicating that the MFD might increase the abundance of the early-life gut LR by improving the maternal gut LR and milk GLN. Furthermore, the *in vitro* fermentation assay with maternal faecal microbiota revealed that the MFD promoted LR proliferation. Therefore, LR and GLN were two main maternal effectors that affected gut health in offspring. Additionally, the explanatory analysis indicated that the main contribution to the gut microbiome in offspring was the combined effects of the maternal gut microbiota and milk. Our previous study reported that the content of glutamate increased after diet fermentation [56]. Thus, the enriched glutamate in the MFD and enhanced milk glutamine metabolism might have contributed to the improved milk GLN. Additionally, the MFD could be easily digested, providing probiotics and their metabolites that might increase the amount of milk GLN. The underlying mechanism of the increase in the abundance of the offspring gut LR might have been attributed to the transfer of the MFD-improved maternal gut LR through the entero-mammary axis [7]. LR and GLN alleviated LPS-induced gut inflammatory disorders by inhibiting p38 MAPK/JNK phosphorylation. The MAPK pathway plays crucial roles in cell physiology and the immune response [57]. The MFD mitigated the increased phosphorylation of p38 MAPK and JNK in the colon during early life, which might have contributed to the reduced colonic inflammation. Ulcerative colitis can be ameliorated by inhibiting MAPK activation and mediating gut barrier integrity [58]. Therefore, it is speculated that the MFD might have alleviated the LPS-induced increase in phosphorylation of p38 MAPK and JNK, thus reducing colonic inflammation in offspring. LR can secrete reuterin and lactic acid to improve gut health and enhance immunity [59]. Although GLN is not generally considered an essential nutrient, it can

improve gut mucosa and barrier functions, and affect the expression of AA receptors and transporters as well as the immune function of the gut [60]. Therefore, the beneficial effects of gut LR and milk GLN on LPS-induced inflammation *in vivo* and *in vitro* were further revealed. Interestingly, LR and GLN together had the best effect, further implying that the combination of maternal milk and gut microbiota plays a major role in regulating the gut health of offspring.

Conclusion

The results revealed that the MFD benefit the maternal gut microbiota and milk metabolites. Meanwhile, the MFD accelerated the maturation of the early-life gut microbiome, promoted the abundance of gut LR, and enhanced microbial functions. In addition, the MFD significantly alleviated LPS-induced colonic inflammation in newborns by suppressing the phosphorylation of p38 MAPK and JNK through the regulation of maternal gut microbiota. Milk GLN and gut LR were two maternal effectors that contributed to gut health in offspring. These results expand our knowledge of pre-emptive strategies for enteric inflammation that target both maternal factors and the early-life gut microbiome and indicate that the MFD is a potential maternal nutritional strategy to regulate gut health in offspring.

Declarations

Ethics approval and consent to participate

The Institutional Animal Care and Use Committee of Zhejiang University approved all experiments involving animals.

Consent for publication

Not applicable.

Availability of data and material

The datasets supporting the conclusions of this article are available in the NCBI Sequence Read Archive (SRA) repository under accession number PRJNA765737, 765800 and 765829.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was funded by China Agriculture Research System of MOF and MARA (CARS-35).

Authors' contributions

The authors' contributions are as follows: CW, MLJ and YZW designed the experiments. CW and SYW conducted the experiments. CW and SYW collected the samples and performed the analysis of samples. CW and SYW analyzed the data. MLJ and YZW help review the manuscript. CW and SYW wrote the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

We thank the staff at the Electronic Microscopy Center in Zhejiang University for their assistance with the TEM and SEM.

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Figures

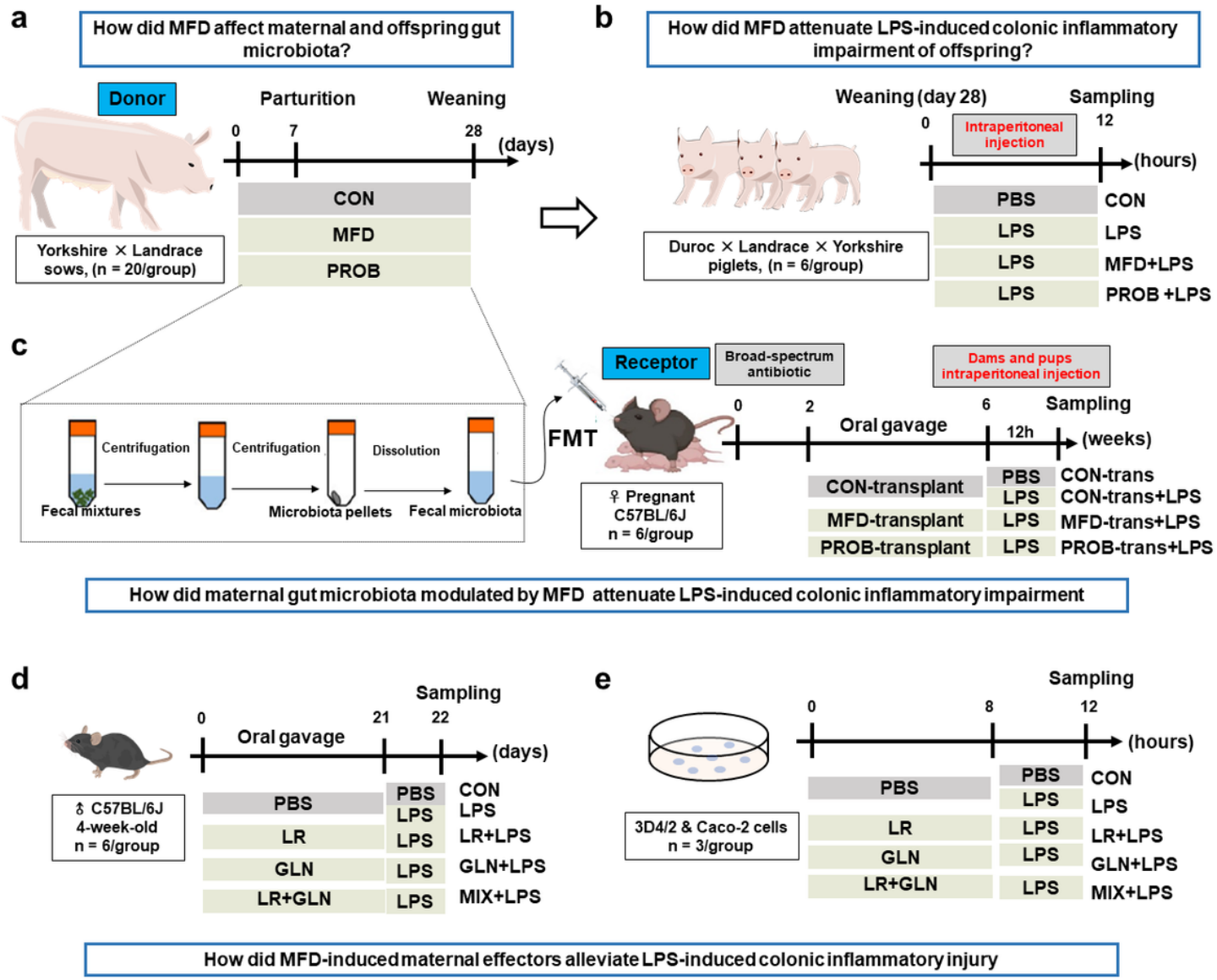

Figure 1

Study design for the whole experiment. (a) Schematic diagram of the fermented diet and probiotics administered to sows. (b) Schematic diagram of the susceptibility of piglets to LPS-induced colonic inflammation. (c) Schematic diagram of the effects of the transplantation of sows' fecal microbiota on the gut health in dams and pups. (c) Schematic diagram of the effects of LR and GLN on the gut health of mice. (e) Schematic diagram of the effects of LR and GLN on inflammation in 3D4/2 and Caco-2 cells. PBS, phosphate buffered saline; LPS, lipopolysaccharides; FMT, fecal microbiota transplantation; LR, Lactobacillus reuteri; GLN, L-glutamine.

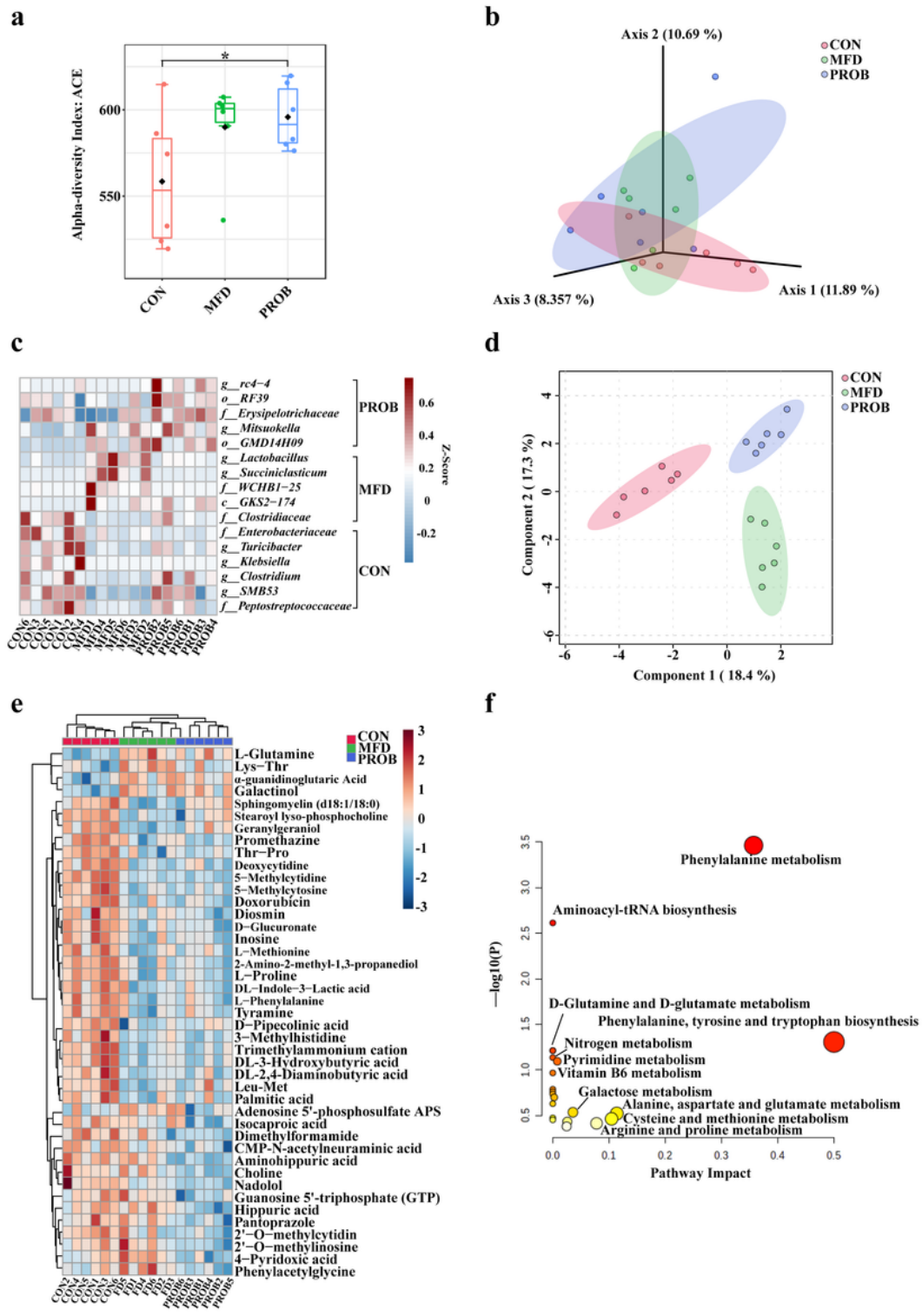

Figure 2

The effects of MFD on the maternal gut microbiome and milk metabolome. (a) ACE index of α diversity. (b) PCoA plots of β diversity based on Bray-Curtis Index (ANOSIM analysis, P < 0.001). (c) Distribution of differential gut genera in maternal individuals (LEfSe analysis, LDA>3.0). (d) sPLS-DA plot of maternal milk metabolites (ANOSIM analysis, P < 0.001). (e) Distribution of differential milk metabolites in

maternal individuals based on Pearson distance and average clustering (VIP>1.0, P<0.05). (f) The significantly different metabolites-related metabolic pathways of milk metabolites.

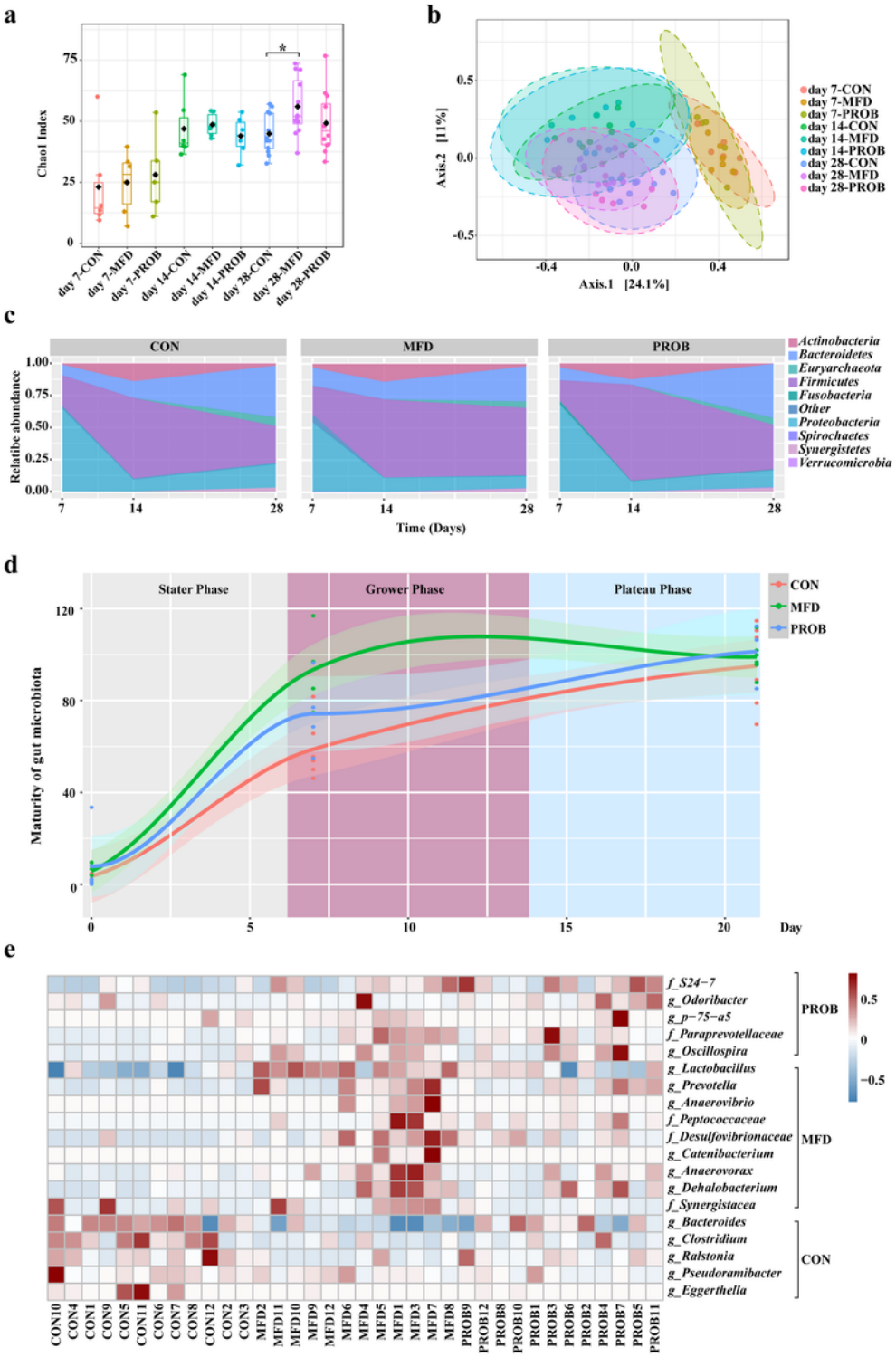

Figure 3

The effects of MFD on the longitudinal and horizontal assembly of early life gut microbiota. (a) Chao1 index of α diversity. (b) PCoA plots of β diversity based on Bray-Curtis Index (ANOSIM analysis, P <

0.001). (c) Area plots at phylum on day 7, 14 and 28. (d) Maturation curve of gut microbiota. (e) Distribution of differential gut genera in offspring individuals on day 28 (LEfSe analysis, LDA>3.0).

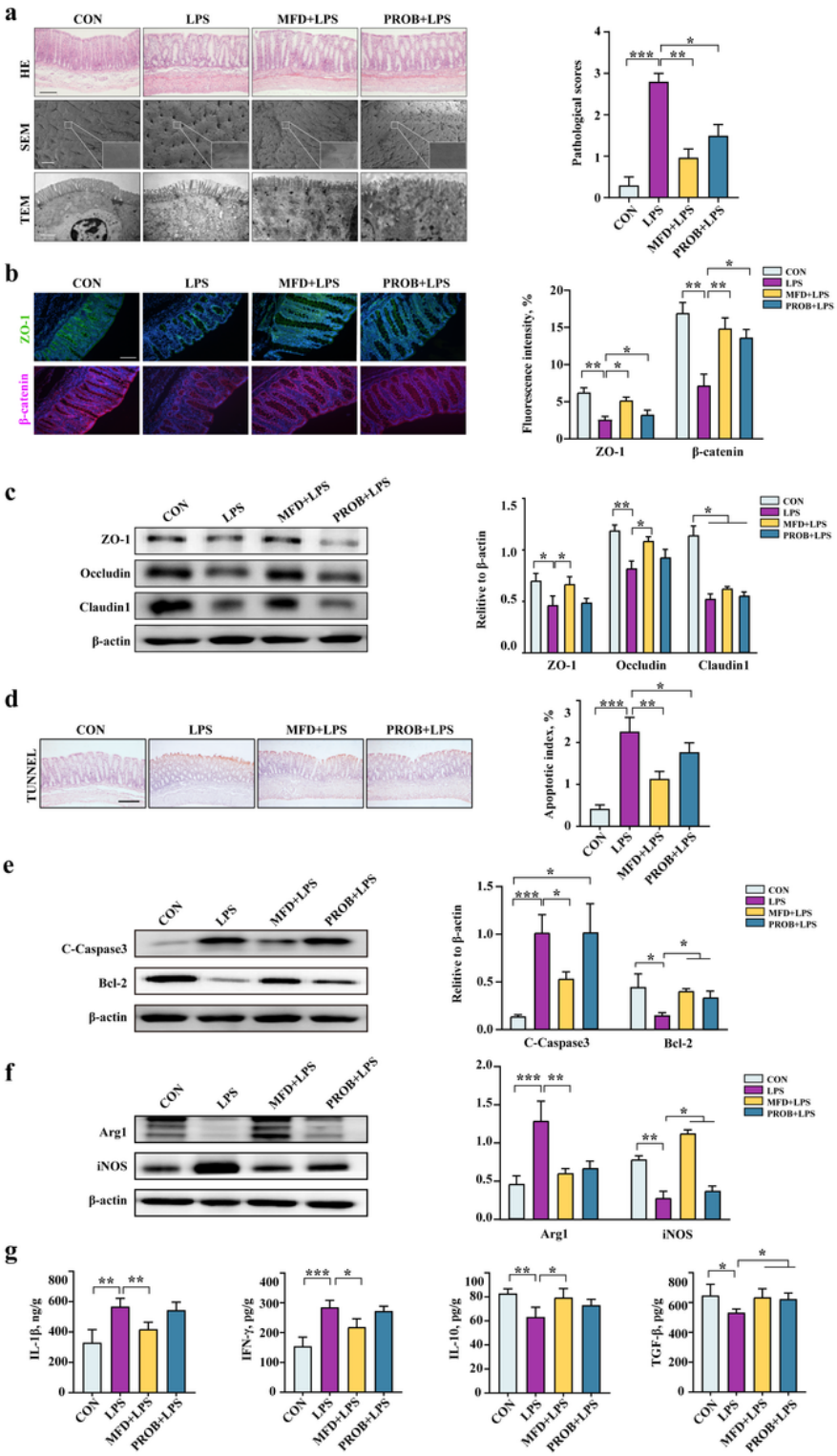

Figure 4

The impacts of MFD on the susceptibilities of offspring to LPS-caused colonic epithelial inflammation. (a) Colonic histological morphology images and pathological scores. Scale bar of H&E: 200 μm; Scale bar of SEM: 1 μm; Scale bar of TEM: 0.2 μm. (b) Images of immunofluorescence of ZO-1 and β-catenin

(c) Colonic tight junction protein expressions. (d) TUNNEL image and statistical analysis. (e) Apoptosis-related protein expression in colon. (f) M1 and M2 macrophage surface protein expression in colon. (g) Colonic cytokines. (h) The phosphorylation of p38, JNK and ERK1/2 in colon. ***: P < 0.001, **: 0.001 < P < 0.01, *: 0.01 < P < 0.05 show the significances, respectively.

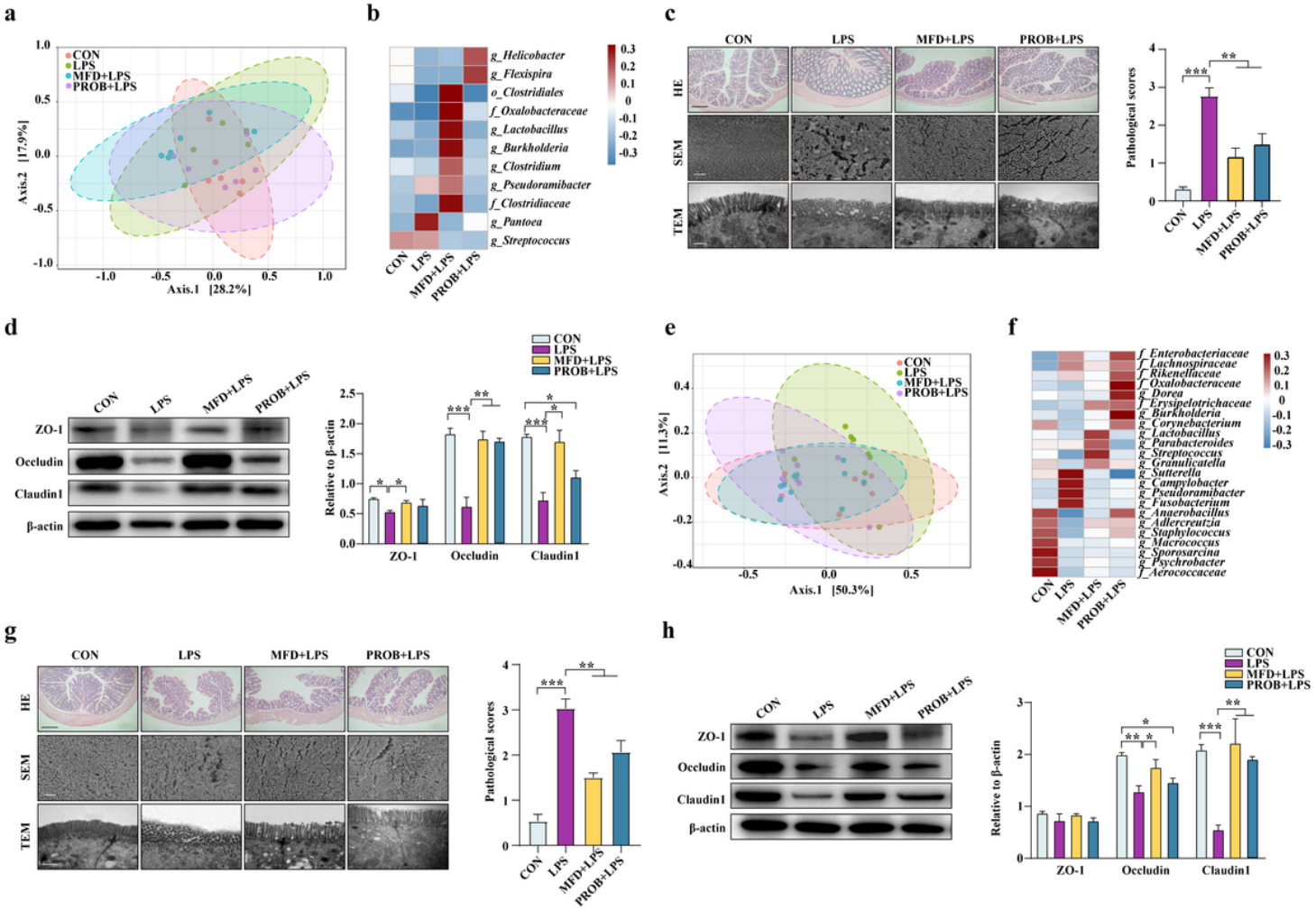

Figure 5

The effects of maternal FMT from MFD on the susceptibilities of dams and pups to LPS-induced colonic epithelial inflammatory impairment. (a) β-diversity of dam gut microbiota based on Bray-Curtis Index (ANOSIM analysis, P=0.049). (b) Distribution of differential genera in dam gut (LEfSe analysis, LDA>3.0). (c) Colonic histological morphology images of dams and pathological scores. Scale bar of H&E: 200 μm; Scale bar of SEM: 1 μm; Scale bar of TEM: 0.2 μm. (d) Colonic tight junction protein expression of dams. (e) β-diversity of pup gut microbiota based on Bray-Curtis Index (ANOSIM analysis, P=0.007). (f) Distribution of differential genera in pup gut (LEfSe analysis, LDA>2.5). (g) Colonic histological morphology images of pups and pathological scores. Scale bar of H&E: 200 μm; Scale bar of SEM: 1 μm; Scale bar of TEM: 0.2 μm. (h) Colonic tight junction protein expression of pups. ***: P < 0.001, **: 0.001 < P < 0.01, *: 0.01 < P < 0.05 show the significances, respectively.

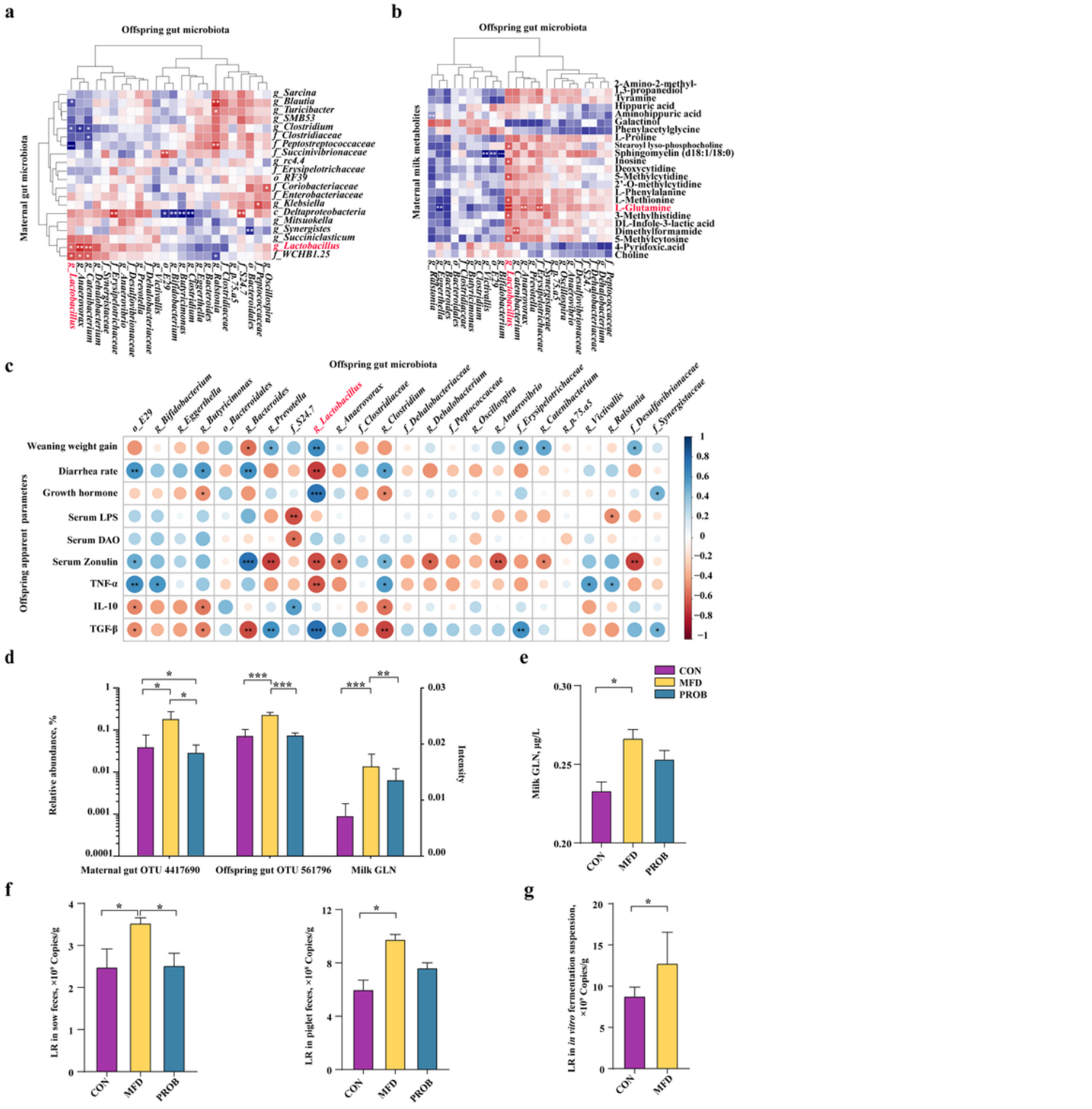

Figure 6

The maternal effectors that contribute to offspring gut health. (a) Correlations between sow gut microbiota and piglet gut microbiota. (b) Correlations between milk metabolites and piglet gut microbiota. (c) Correlations between piglet gut microbiota and piglet serum indexes. (d) The relative abundance of the most differential OTU in both sow and piglet gut microbiota and the intensity of GLN in the milk. (e) The absolute quantity of fecal LR of sow and piglets. (f) The absolute quantity of

supernatant LR of MFD in vitro fermentation system. (g) The absolute concentration of milk GLN. ***: P < 0.001, **: 0.001 < P < 0.01, *: 0.01 < P < 0.05 show the significances, respectively.

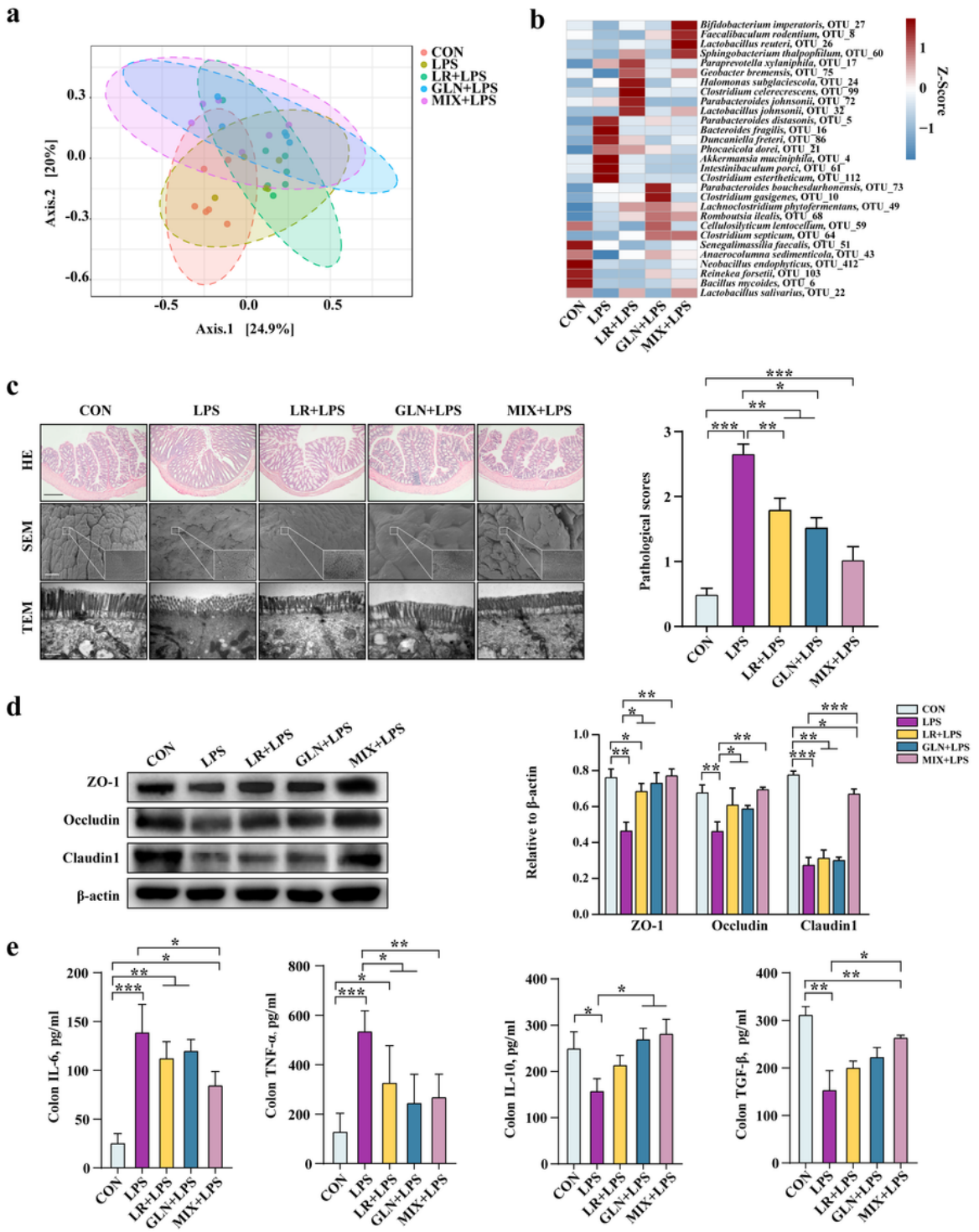

Figure 7

The validation of the effects of LR and GLN on LPS-induced colonic inflammatory impairment on mice. (a) β-diversity of gut microbiota based on Bray-Curtis distance (ANOSIM analysis, P<0.001). (b) Distribution of differential gut genera in mice (LEfSe analysis, LDA>3.0). (c) Colonic histological

morphology images and pathological scores. Scale bar of H&E: 200 μm; Scale bar of SEM: 1 μm; Scale bar of TEM: 0.2 μm. (d) Colonic tight junction protein expression. (e) Colonic cytokines. ***: P < 0.001, **: 0.001 < P < 0.01, *: 0.01 < P < 0.05 show the significances, respectively.

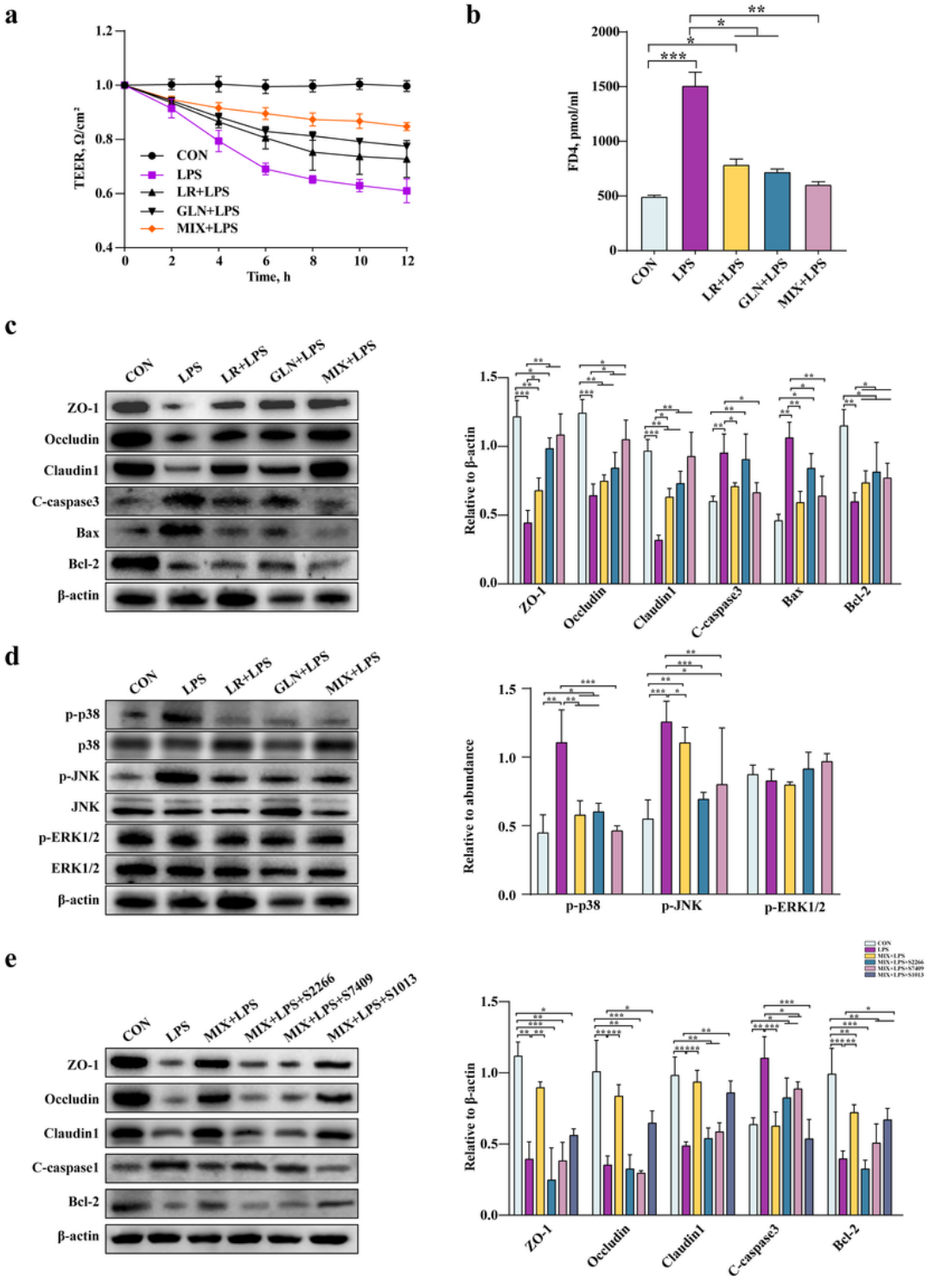

Figure 8

The validation of the effects of LR and GLN on LPS-induced colonic inflammatory impairment on cells. (a) Changes in the TEER values of Caco-2 cells for the completely differentiated monolayer; (b) FD4

content in lower compartments of Transwells. (c) Tight junction and apoptosis-related protein expression of Caco-2. (d) p38, JNK and ERK1/2 phosphorylation of Caco-2. (e) The tight junction and apoptosis-related protein expression when Caco-2 cells were treated with p38, JNK and ERK1/2 specific activators. ***: P < 0.001, **: 0.001 < P < 0.01, *: 0.01 < P < 0.05 show the significances, respectively.

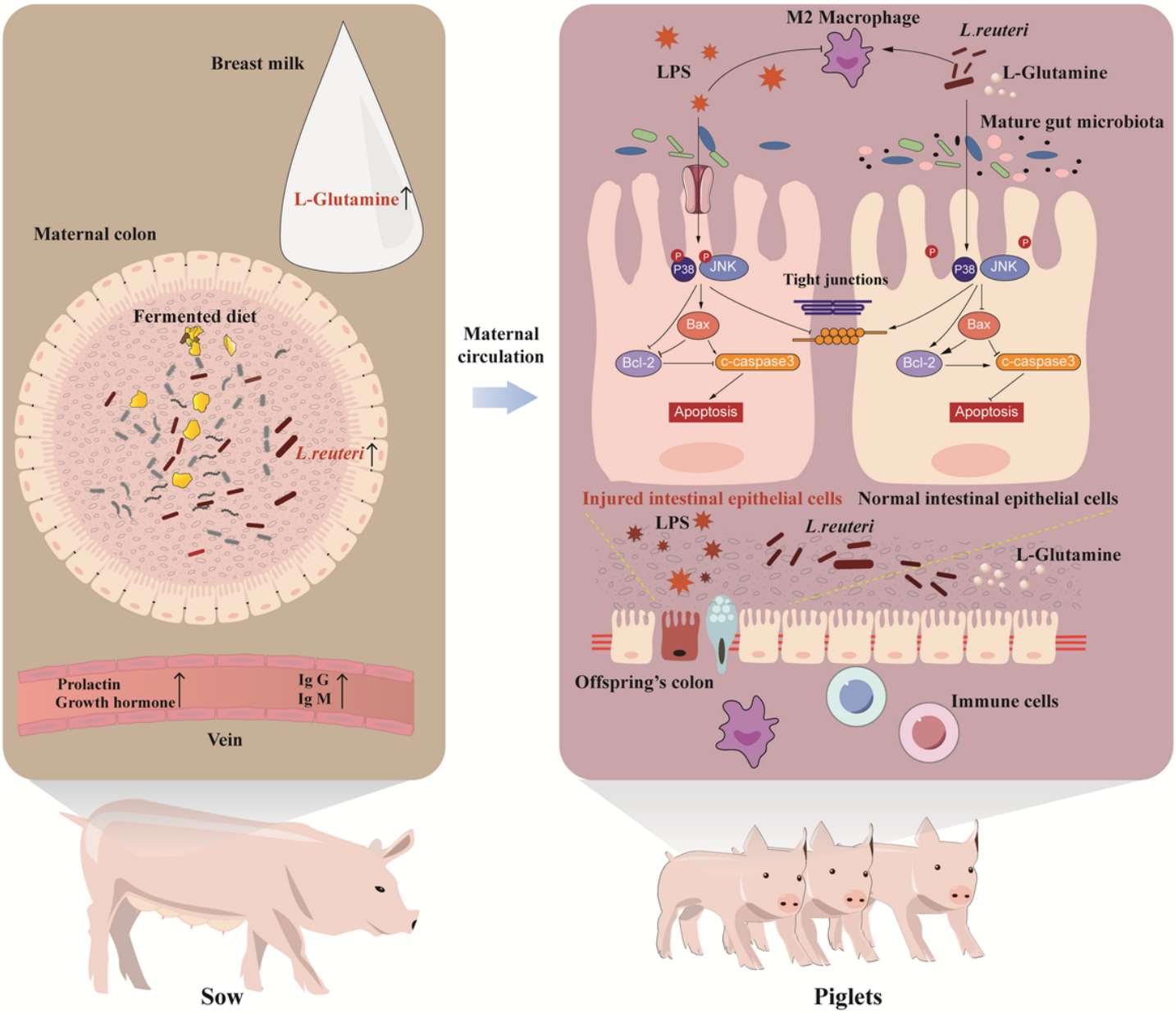

Figure 9

The mechanism of MFD drives early life gut health.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SFigureSTable.docx
- TableS3.Differentialmilkmetabolites.csv